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The effect of 42 different compounds on red cell ATP and 2,3-DPG levels was studied. Of these oxalate, glyoxalate, ethyl oxaloacetate and L-phenylalanyl-L-alanine were found to improve 2,3-DPG levels. Glyoxalate and ethyl oxaloacetate exert their effect by conversion to oxalate. The latter compound has been shown to inhibit pyruvate kinase and in this way modifies red cell metabolism so as to cause an increase in red cell 2,3-DPG levels. L-phenylalanyl-L-alanine is rapidly hydrolyzed to phenylalanine and alanine, both of which also inhibit pyruvate kinase.

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METABOLIC REGULATION OF 2,3-DPG IN STORED RED BLOOD CELLS

FINAL REPORT

SHOBHANA VORA

MARCH 1, 1988

Supported by

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1. Problem under study. The investigations carried out under this contract were aimed at attempting to find better mechanisms for maintenance of red cell 2,3-DPG levels during storage.

2. Background and Review. Since the studies of Benesch and Benesch (1) and Chanutin and Curnish (2) showing that the oxygen affinity of hemoglobin was strongly influenced by 2,3 phosphoglycerate (2,3-DPG) levels there has been a great deal of interest in attempting to improve the stability of 2,3-DPG levels during storage. This problem has been reviewed extensively previously by the principal investigator (3-6) and by others (7-9).

3. Rationale used in current study. The basic premise underlying the current studies was that inhibitors of glycolysis, particularly at the pyruvate kinase step, could exert an affect on the maintenance of 2,3-DPG levels.

4. Experimental Methods. In the initial screening studies blood from normal volunteer donors was drawn into CPD (citrate-phosphate-dextrose) or CPDA1 (citrate-phosphate-dextrose-adenine) solution (Fenwal Laboratories). The blood was divided into 4 nearly equal portions and was distributed into plastic blood bags. The volume of each aliquot was measured by weighing the bag and minor differences in volume were corrected by addition of 0.9% NaCl solution. Various substances were added in the saline to each of the aliquots. Samples without additions served as controls. ATP and 2,3-DPG estimations were carried out weekly or semiweekly for at least 6 weeks using standard methods (10).

In subsequent studies, 450 ml blood was drawn into 63 ml CPDA1, divided into 95 or 99 ml portions and distributed into plastic blood bags. The substance under study was added in 1 ml or 5 ml 0.9% NaCl to each 95 or

99 ml aliquot of blood. Saline without preservative was added to the control.

The blood samples were incubated at 37°C for 1 to 1-1/2 hr. prior to storage. The internal pH of red cells was measured by freezing and thawing packed red cells and measuring the pH of the lysate at 0°C with a Corning model 150 pH meter. Oxalate determinations (11) were carried out on plasma ultrafiltrates made from blood with added glyoxalate before storage and after 1 week.

5. Results. Table 1 lists the 42 substances screened and an evaluation of their affect on ATP and 2,3-DPG levels during storage.

Inspection of these data indicated that oxalate, glyoxalate, ethyl oxaloacetate, L-phenylalanyl-L-alanine, phenylalanine t-butyl ester, and aminoisobutyric acid might be effective in the improving maintenance of 2,3 DPG concentrations.

Accordingly, the effects of these compounds was reinvestigated, and the effect of oxalate, glyoxalate, ethyl oxaloacetate, and L-phenylalanyl-L-alanine on the maintenance of 2,3 DPG were found to be reproducible. Representative experiments are shown in figures 1 and 2.

To determine whether the effect of glyoxalate might be due to its conversion to oxalate, previously known to exert an effect on 2,3-DPG levels (12,13), the oxalate contents of blood samples stored with and without oxalate were determined. The concentration of oxalate in control blood was less than 5 moles. In contrast, the concentration of oxalate in blood stored with glyoxalate rose rapidly, reaching a 500 mole concentration within one hour of collection. Conversion of glyoxalate to oxalate required the presence of red cells. Glyoxalate added to plasma would not convert it to oxalate.

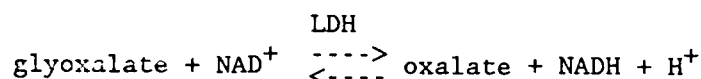
Amino acid analysis of blood stored with L-phenylalanyl-L-alanine showed complete hydrolysis of the dipeptide to phenylalanine and alanine within a week of storage.

6. Discussion and Conclusions. A large number of compounds have been tested for their effect on erythrocyte 2,3-DPG and ATP levels of blood stored in citrate phosphate adenine solutions. Inhibitors of ATPase, such as ouabain did not affect ATP levels. This finding is not surprising, since red cell ATPase is scarcely active at 4°C (14,15). Neither was an effect observed with most of the other compounds tested.

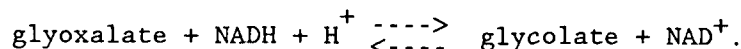
Four compounds were found to be effective in raising 2,3-DPG levels. These were oxalate, glyoxalate, ethyl oxaloacetate and L-phenylalanyl-L-alanine. The effect of oxalate was first discovered by Kandler et al (13) when he investigated the putative effect of ascorbate on 2,3-DPG levels. Pure ascorbate, formerly thought to be a potent agent in preserving 2,3-DPG (16) was without effect, but oxalate, contaminating the ascorbate was found to be the active substance. Recently we have shown that even .05 mM oxalate has a substantial effect on red cell 2,3-DPG levels and that the effect of this substance is largely due to inhibition of pyruvate kinase (12). Ethyl oxaloacetate is known to hydrolyze to oxalate, acetate, and ethanol (17).

The effect of glyoxalate was more of a surprise. Tested because of its similarity to glycolate and oxalate (Fig. 3), glyoxalate differs from oxalate only by a single oxidation state. Glyoxalate is a 2-carbon analogue of pyruvate while glycolate may be regarded as the corresponding 2-carbon analogue of lactate. Red cell membranes are freely permeable to the glyoxalate anion (18). In the presence of NAD, glyoxalate is oxidized to oxalate by heart (19) and muscle (20) lactate dehydrogenase. The same enzymes also have the capability of reducing glyoxalate to glycolate in the

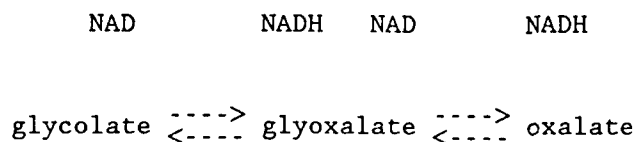
presence of NAD. Oxalate, increases 2,3-DPG levels of stored red cells, and the quantity formed from glyoxalate is clearly sufficient to account for the effect of glyoxalate on 2,3-DPG levels. Oxalate appeared rapidly in blood to which glyoxalate is added. Presumably it is formed by oxidation of glyoxalate in the reaction:



For this reaction to create 0.5 mM oxalate, the concentration that was achieved within an hour of blood collection, the NADH formed must be re-oxidized to  $\text{NAD}^+$ . There is insufficient pyruvate in blood to serve as an oxidizing agent for the amount of  $\text{NAD}^+$  required. It may well be that a portion of the glyoxalate itself regenerates the need for  $\text{NAD}^+$  in the reaction:



The overall reaction, then, is:



Thus, the effect of glyoxalate seems to be due simply to its coupled oxidate to oxalate. L-phenylalanyl-L-alanine had a pronounced effect on 2,3-DPG levels after 1 week's storage, but none subsequently. It is of interest that high concentrations of L-alanine (21,22) and L-phenylalanine (22) have previously been shown to aid in 2,3-DPG preservation in stored red cells. The red cell is rich in peptidase activity (23) and is nearly



impermeable to those dipeptides that have been tested.

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TABLE I. The Effect of Various Additives on the ATP and 2,3 DPG Levels of Stored Blood

Preservative	Additive	Concentration (mM)	ATP	2,3-DPG
CPD	glyoxylate	1	↓	↑
CPD	α-aminoisobutyric acid	1	↑	↑
CPD	cysteine*	5	-	-
CPD	3-I-butyl-1-methyl-xanthene	3	-	-
CPD	oxalate	2	↓	↑
CPDA1	palmitic acid	0.2	-	-
CPDA1	myristic acid	0.2	-	-
CPDA1	hydroxypyruvate	2	↑	-
CPDA1	ethyl oxaloacetate	2	↑	↑
CPDA1	keto glutarate	5	-	-
CPDA1	phenyl pyruvate	2	-	-
CPDA1	isocitrate	1	-	↓
CPDA1	t-aconitate	1	↑	↓
CPDA1	malonate	5	-	↓
CPDA1	oxaloacetate	2	-	↓
CPDA1	phenylalanine methyl ester	4.3	-	-
CPDA1	phenylalanyl methionine	2	-	-
CPDA1	oxobutyrate	1	↑	-
CPDA1	cysteine methyl ester	2	-	-
CPDA1	maleate	2.5	-	-
CPDA1	malate	2.5	-	-

TABLE I (Continued)

Preservative	Additive (mM)	Concentration ATP		2,3-DPG
CPDA1	tartrate	2.5	-	-
CPDA1	cupric chloride	0.002	-	-
CPDA1	nickel sulfite	0.005	-	-
CPDA1	cobalt sulfate	0.005	-	-
CPDA1	valine	5	-	-
CPDA1	proline	5	-	-
CPDA1	tryptophan	5	-	-
CPDA1	D-alanine	5	†	-
CPDA1	isoleucine	5	-	-
CPDA1	methionine	5	†	-
CPDA1	L-phenylalanyl-L-alanine	5	†	†
CPDA1	oleate	0.05	-	-
CPDA1	linoleate	0.05	-	-
CPDA1	nicotinate	1	-	-
CPDA1	nicotinaldehyde	1	-	-
CPDA1	quinolinate	1	-	-
CPDA1	glycolaldehyde	1	-	-
CPDA1	nicotinamide	40	-	-
CPDA1	methyl glyoxal	1	-	-
CPDA1	Quabain	0.2	-	-
CPDA1	Quercitin	0.005	-	-

† &gt;25% increase

↓ &gt;25% decrease

\* amino acids are in the L-conformation unless otherwise indicated



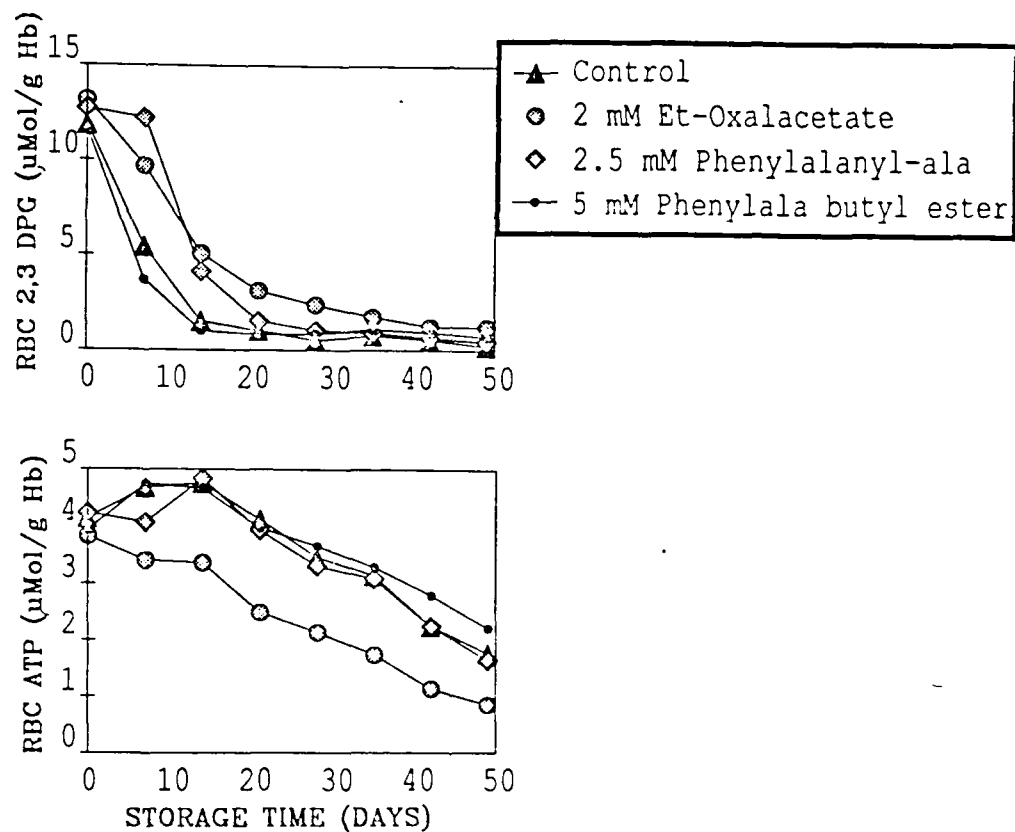


FIGURE 1 The effect of storage of packed red cells with the addition of ethyloxalacetate (Et-oxalacetate) L-phenylalanyl-L-alanine (phenylalanyl-ala) and phenylalamine butyl ester (phenylala butyl ester) at the final concentrations shown on ATP and 2,3-diphosphate glycerate levels.

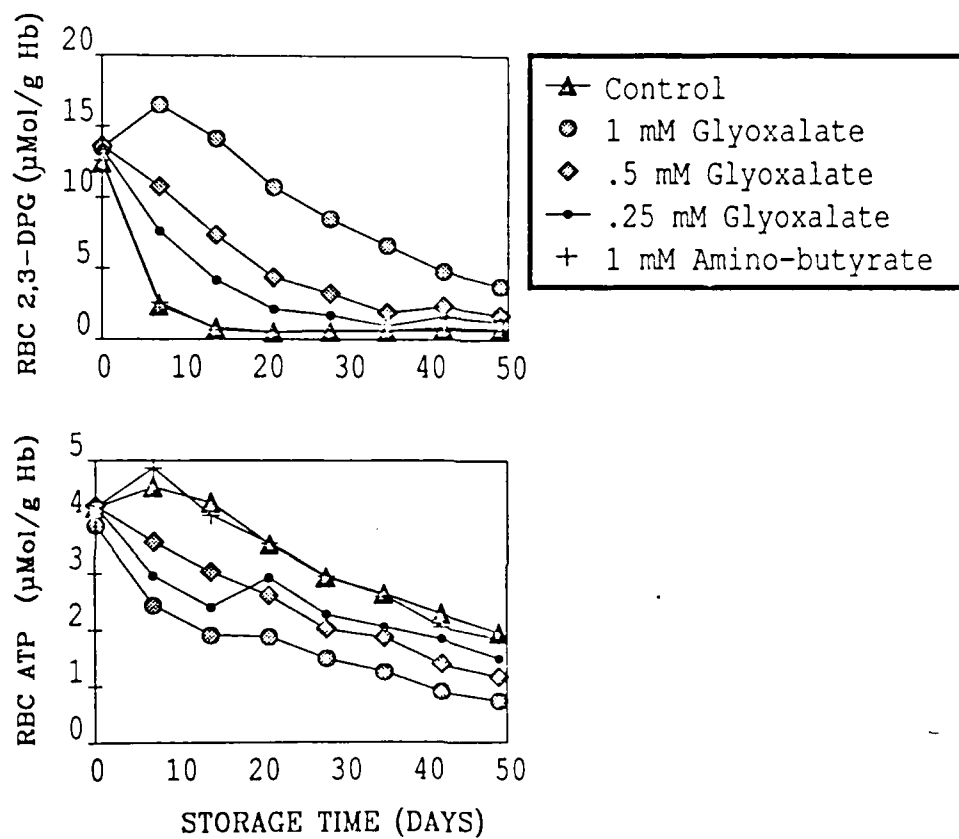
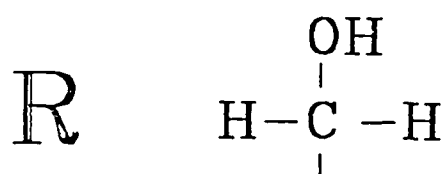
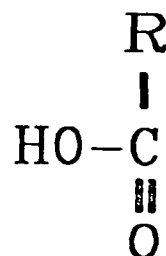
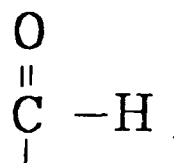


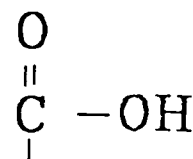
FIGURE 2 The effect of storage of red cells with the addition of  $\alpha$ -amino isobutyric acid (Amino-butyrate) and glyoxalate at the final concentrations shown on red cell ATP and 2,3-DPG levels.



*GLYCOLATE*



*GLYOXALATE*



*OXALATE*

FIGURE 3 The structural relationship between glycolate, glyoxalate, and oxalate. All three compounds are 2-carbon carboxylic acids, differing from each other only in their state of oxidation. They are interconvertible through the lactate dehydrogenase reaction (see text).